PB84-101518

Enumeration and Morphological Characterization of Bacteria Indigenous to Subsurface Environments

Cornell Univ., Ithaca, NY

Prepared for

Robert S. Kerr Environmental Research Lab.
Ada, OK

1983

U.S. Department of Commerce
National Technical Information Service
NTIS

TECHNICAL REPORT DATA
(Please read Instructions on the reverse before completin

1. REPORT NO.	2.	3. R
EPA-600/J-83-043		PB84-101518

4. TITLE AND SUBTITLE	5. REPORT DATE
Enumeration and Morphological Characterization of Bacteria Indigenous to Subsurface Environments (Journal Version)	1983
	6. PERFORMING ORGANIZATION CODE

7. AUTHOR(S)	8. PERFORMING ORGANIZATION REPORT NO.
W. C. Ghiorse[1] and D. L. Balkwill[2]	

9. PERFORMING ORGANIZATION NAME AND ADDRESS	10. PROGRAM ELEMENT NO.
[1]Dept. Microbiology Cornell University Ithaca, NY 14853 [2]Dept. Microbiology University of New Hampshire Durham, NH 03824	CBPC1A
	11. CONTRACT/GRANT NO.
	CR-806931

12. SPONSORING AGENCY NAME AND ADDRESS	13. TYPE OF REPORT AND PERIOD COVERED
Robert S. Kerr Environmental Research Lab. - Ada, OK U.S. Environmental Protection Agency Post Office Box 1198 Ada, OK 74820	14. SPONSORING AGENCY CODE EPA/600/15

15. SUPPLEMENTARY NOTES

REF: Developments in Industrial Microbiology, 24:213-224, 1983

16. ABSTRACT

Subsurface samples from a rapid infiltration wastewater system under construction at Ft. Polk, LA were examined by employing aseptic sampling devices, acridine orange (AO)-fluorescent cell counts, and transmission electron microscopy (TEM). Samples contained 1 to 10 million AO-fluorescent bacteria per g dry wt. The Ft. Polk samples also contained forms that may have been eukaryotic microorganisms. Plate counts were very low, usually less than 100 colony forming units (CRU) per g dry wt. Since total organic carbon (TOC) was invariably less than 0.1% in the samples, attempts were made to cultivate the indigenous bacteria on low-nutrient media; but very few isolates were obtained. Indigenous bacteria were observed by AO-fluorescence microscopy, however. They were morphologically diverse and frequently were found in groups (microcolonies). TEM of the indigenous microflora revealed a greater proportion of Gram-positive than Gram-negative bacteria. Many of these bacteria contained PHB-like storage inclusions; some were enmeshed in glycocalyx-like material.

These results clearly indicate that a variety of bacteria exist at uniformly low population density in the subsurface environments examined at Ft. Polk. They also suggest that subsurface bacteria are adapted to survive under stress resulting from the low concentrations of nutrients found in the subsurface, but not be able to endure elevated levels of toxic pollutants which may accumulate in their environment.

17. KEY WORDS AND DOCUMENT ANALYSIS

a. DESCRIPTORS	b. IDENTIFIERS/OPEN ENDED TERMS	c. COSATI Field/Group
Microbiology Bacteria Ground Water Subsurface Investigations	Soil Microbiology Soil Environments	68 D

REPRODUCED BY
NATIONAL TECHNICAL
INFORMATION SERVICE
U.S. DEPARTMENT OF COMMERCE
SPRINGFIELD, VA 22161

18. DISTRIBUTION STATEMENT	19. SECURITY CLASS *(This Report)*	21. NO. OF PAGES
Release to the public.	Unclassified	13
	20. SECURITY CLASS *(This page)*	22. PRICE
	Unclassified	

EPA Form 2220-1 (9-73)

NOTICE

This document has been reviewed in accordance with U.S. Environmental Protection Agency policy and approved for publication. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

Reprinted from Volume 24 of DEVELOPMENTS IN INDUSTRIAL MICROBIOLOGY
A Publication of the Society for Industrial Microbiology - 1983

EPA-600/J-83-043
JOURNAL ARTICLE

CHAPTER 16

Enumeration and Morphological Characterization of Bacteria Indigenous to Subsurface Environments

W. C. GHIORSE AND D. L. BALKWILL

Department of Microbiology, Cornell University, Ithaca, New York 14853 and Department of Microbiology, University of New Hampshire, Durham, New Hampshire 03824

Previous work employing aseptic sampling devices, acridine orange (AO)-fluorescent cell counts, and transmission electron microscopy (TEM) revealed uniformly low numbers of bacteria and a lack of eukaryotic microorganisms in uncontaminated subsurface samples from two Oklahoma aquifers. Plate counts indicated that as many as one-half of the bacteria in these samples were viable and microcosm studies indicated that the indigenous microflora could degrade toluene and chlorobenzene.

In the present work, subsurface samples from a rapid infiltration waste-water system under construction at Ft. Polk, LA, were examined by similar methods. Like the Oklahoma samples, those from Ft. Polk contained 1 to 10 million AO-fluorescent bacteria per gram dry weight. The Ft. Polk samples, however, contained forms that may have been eukaryotic microorganisms. Plate counts on these samples were very low, usually less than 100 colony-forming units (CFU) per gram dry weight. Since total organic carbon (TOC) was invariably less than 0.1% in the samples, attempts were made to cultivate the indigenous bacteria on low-nutrient media; but very few isolates were obtained. Indigenous bacteria were observed by AO-fluorescence microscopy. They were morphologically diverse and frequently were found in groups (microcolonies). The TEM of the indigenous microflora revealed a greater proportion of gram-positive than gram-negative bacteria. Many of these bacteria contained PHB-like storage inclusions and some were enmeshed in glycocalyx-like material. These results indicate that a variety of bacteria exists at uniformly low population density in the subsurface environments examined. They also suggest that subsurface bacteria are adapted to survive under stress resulting from the low concentrations of nutrients found in the subsurface. Finally, it is proposed that sparse populations of subsurface bacteria under low-nutrient stress may not be able to endure elevated levels of toxic pollutants which may accumulate in their environment.

INTRODUCTION

Subsurface environments can be defined as those which occur beneath true soil zones of the earth's crust. They include unsaturated zones above water tables as well as saturated zones (aquifers) containing groundwater. Shallow aquifers are used widely for irrigation and drinking water. In recent years, they have become increasingly contaminated with pollutants leaching from landfills and toxic-waste storage sites. Deep aquifers are used as sources of water and have been in danger of contamination from liquid waste since the practice of deep-well injection began several years ago. Thus, the dual problems of toxic-waste disposal and concomitant groundwater pollution recently have caused considerable concern among industrialists, environmentalists, and governmental agencies alike. This concern recognizes that almost no information exists concerning the ability of subsurface environments to copy with pollutants.

One way an environment can cope with organic pollutants is by biodegradation resulting principally from microbial activity (Alexander 1981). Conventionally, it has been assumed that microorganisms in surface and subsurface soils are capable of degrading many of the substances that may pollute groundwater. However, as previously discussed (Dunlap and McNabb 1973; Ghiorse and Balkwill 1981; McNabb and Dunlap 1975), although substantial evidence supports this assumption with regard to surface soil, evidence indicating that microorganisms are present and active in subsurface soil, is at best, presumptive.

Most earlier studies on subsurface microorganisms have been hampered by the technical difficulties and great expense of obtaining samples not contaminated by surface microbes. Thus, because of the ever-present possibility of surface contamination, the results of most previous studies on subsurface microorganisms are equivocal (see, for example, Olson et al. 1981; Willis et al. 1975).

Using aseptic coring devices and special handling procedures developed at the R. S. Kerr Environmental Research Laboratory, Ada, OK (Dunlap et al. 1977), it is now possible to obtain subsurface samples that are not contaminated. Employing these devices and procedures, the first uncontaminated samples were obtained in 1981 from two shallow aquifers in Oklahoma. These samples were examined by AO-fluorescence microscopy, TEM, cultural techniques, and microcosm studies to determine the types, abundance, and activities of microorganisms in them. Remarkably constant numbers of bacteria, between 1 and 10 million/g dry wt, and no eukaryotic microorganisms were found in the samples (Ghiorse and Balkwill 1981). In some cases, nearly half of the indigenous bacteria were viable, as indicated by their ability to grow on culture media prepared from diluted extracts of subsurface soil (Balkwill and Ghiorse 1982). Furthermore, the industrial pollutants toluene and chlorobenzene were degraded biologically in microcosms prepared from the samples. However, other pollutants, such as halogenated ethanes and ethylenes, were not degraded (Wilson et al. 1983b).

The present report describes the enumeration and morphological characterization of microorganisms in another set of uncontaminated subsurface samples obtained at Fort Polk, LA, where a rapid infiltration waste-water treatment system is under construction. The samples were obtained before the application of waste water to provide a basis for comparison with samples to be taken after the system is in operation. Studies are reported in the two following chapters of this volume on biotransformations of organic pollutants (Wilson et al. 1983a) and on microbial biomass, community composition, and nutritional status (White et al. 1983) in samples from the same site.

Materials and Methods

Description and Handling of Subsurface Samples

Two boreholes (6B and 7) were drilled adjacent to a newly constructed holding basin at the Ft. Polk rapid infiltration site. Uncontaminated samples were obtained in both boreholes from two levels in the unsaturated zone and from one level in the saturated zone (see Table 1) employing an autoclaved core barrel and aseptic handling procedures as described by Wilson et al. (1983a). Borehole 6B was located on a dike

TABLE 1. *Numbers of bacteria in subsurface samples from Ft. Polk, LA.*

		AO-Fluorescent Cells (S.D.)/g dry wt		
Borehole	Depth, m	Fixed[a]	Stored[b]	CFU[b,c]/g dry wt
6B	3.7 (U)	$1.6(5.1) \times 10^6$	$3.4(1.7) \times 10^6$	$<10^2$
6B	5.5 (U)	$2.2(0.8) \times 10^6$	$1.3(1.0) \times 10^6$	$<10^2$
6B	6.7 (S)	$2.7(2.8) \times 10^6$	$3.0(2.8) \times 10^6$	$<10^2$
7	1.2 (U)	$1.5(0.3) \times 10^7$	$7.0(7.8) \times 10^6$	1×10^5
7	4.0 (U)	No data	$1.3(1.7) \times 10^6$	$<10^2$
7	5.1 (S)	$8.9(7.6) \times 10^6$	$9.8(0.7) \times 10^6$	$<10^2$

U = unsaturated zone.
S = saturated zone.

[a]Samples were fixed with glutaraldehyde at time of sampling then shipped to the laboratory for AO-fluorescent cell counts.

[b]Samples were cooled immediately at 0 to 4 C, then shipped to the laboratory. They were stored at 4 C until plate counts for AO-fluorescent cell counts were made.

[c]Only the colony counts of aerobically incubated SEA from a single plating are reported. Similar results were obtained when PYG and 1% PYG media were used. A second plating after 6 mo. of storage at 4 C gave similar results. Colony counts on plates incubated anaerobically were always lower than on those incubated aerobically.

approximately 2 m high and borehole 7 was located just off the dike.

Borehole 7 samples contained between 77% and 91% sand, 2% and 22% silt, and 7% and 15% clay. The TOC in these samples was 0.053% at 1.2 m, 0.022% at 4.0 m, and 0.016% at 5.1 m. The pH ranged from 4.1 to 4.9. Borehole 6B samples were also sandy in texture and low in TOC (0.020% at 3.7 m, 0.019%% at 5.5 m, and 0.015% at 6.7 m). Their pH ranged from 4.1 to 4.2.

Freshly collected samples were cooled immediately at 0 to 4 C and then shipped cold to Ithaca, NY and Durham, NH, for microscopic examination and enumeration of microorganisms. Some samples were fixed before shipping by diluting them in a solution of 2.5% (v/v) glutaraldehyde dissolved in 0.1% (w/v) sodium pyrophosphate.

AO-fluorescent cell counts. A modification of Trolldenier's (1973) epifluorescence method was used to count total numbers of microbial cells in both fresh and fixed samples. In the modified method, 2.5 g of fresh soil was diluted in 22.5 ml of filter-sterilized 0.1% sodium pyrophosphate and shaken for 30 min at 160 rpm. The resulting soil suspension was decanted into 25-ml scintillation vial and allowed to settle for 2 min. After settling, a 9-ml portion was removed from the top of the vial, mixed with 1 ml of molten 1% (w/v) agar, then fixed, if necessary, by adding 0.1 ml of a 50% (v/v) glutaraldehyde solution. Subsamples, usually 5 or 10 μl, were smeared over a 1 cm^2 area on a glass slide and air dried. The smears were stained with 0.01% (w/v) acridine orange and examined in a

Zeiss Standard 18 microscope equipped for phase contrast and epiillumination as described previously (Ghiorse and Balkwill 1981). Representative fields in stained smears were photographed on Kodak Ektachrome T160 color film. For black and white reproduction, color slides were copied on Kodak Tri X film.

Green, fluorescent particles of bacterial size and shape were counted in at least 10 separate stained smears from each 2.5 g of fresh soil. The AO-fluorescent cell counts per g of over-dried (105 C) soil were calculated from the mean number of green, fluorescent cells in at least 50 microscope fields, using appropriate conversion factors. Samples that had been fixed before shipping also were diluted and counted as described above except that the amounts of starting material and diluent were proportionally greater.

Counts from nonsettled samples were not significantly different from those that were allowed to settle for 2 min before adding agar. The settling step in this procedure allows a more homogenous subsample to be counted. In some of these samples many irregularly shaped particles were observed that fluoresced green after AO staining. Since these particles were easily distinguished from bacteria on the basis of size and shape, they did not affect the AO-fluorescent cell counts.

Plate counts. Numbers of viable microorganisms in fresh samples were estimated by a plate-count technique as follows. Ten grains of subsurface material were blended with 100 ml of sterile 0.1% sodium pyrophosphate for 1 min in a Waring Blendor to form a soil suspension. Aliquots of the serially diluted suspension were spread in triplicate on SEA, PYG, and 1% PYG plating media. The SEA medium contained the following ingredients per liter: soil extract, 100 ml; distilled water, 900 ml; agar, 15 g. Soil extract was made by autoclaving a 1:2 (w/v) suspension of the appropriate subsurface material in distilled water for 1 h, after which the liquid extract was clarified by centrifugation and filtration. The PYG medium contained the following ingredients per liter: peptone, 5 g; glucose, 10 g; trypticase, 5 g; yeast extract, 10 g; agar, 15 g. The 1% PYG medium was a 1:100 dilution of PYG medium. Plates were incubated aerobically at 25 C. Colonies that grew on the plates were counted after 1 to 2 wk of incubation. Duplicate sets of plates from some samples were incubated anaerobically. Since the numbers of colonies on the anaerobic plates were always much lower than on the aerobic plates, plates were not routinely incubated anerobically.

Cultivation of Subsurface Microorganisms

Since most of the plate counts on these samples were very low (Table 1), attempts were made to cultivate and isolate microorganisms employing a method that previously had been successful for isolating bacteria from the Oklahoma subsurface samples (W. Hahn, pers. commun.). In this method, 1 g of subsurface soil was added to 10 ml of a pH 7.0 buffered salts solution containing per liter: 6.4 g NaCl, 0.16 g KCl, 0.92 g K_2HPO_4, 0.16 g KH_2PO_4, 0.13 g $CaCl_2 \cdot H_2O$, and 0.10 g $MgCl_2 \cdot 6H_2O$. The mixture was shaken at 160 rpm on a rotary shaker for 30 min. One-tenth milliliter of the mixture was then spread on a medium prepared from an extract of Ft. Polk subsurface soil as described above, except that a 1:1 (w/v) suspension of soil and distilled water was autoclaved initially. The pH of the extract was 6.3. Colonies

that appeared on the Ft. Polk SEA plates were transferred to fresh SEA medium and to a dilute PYG medium containing per liter: peptone, yeast extract, and glucose, 0.25 g each; $MgSO_2 \cdot 7H_2O$, 0.6 g; $CaCl_2 \cdot H_2O$, 0.07 g; agar, 15 g. The inoculated plates were incubated aerobically at 22-25 C.

Transmission Electron Microscopy

Microorganisms were released from subsurface soil and concentrated for TEM thin-sectioning by a variation of a previously described blending-centrifugation technique (Balkwill et al. 1977; Ghiorse and Balkwill 1981) as follows. A 10-g sample of subsurface material was suspended for prefixation in 100 ml of a 3.0% (v/v) glutaraldehyde solution in 0.1% sodium pyrophosphate. After overnight prefixation, microbial cells were released from mineral particles by blending the prefixed suspension for 1 min in a Waring Blendor. The blended suspension was transferred immediately to a 100-ml graduate cylinder and allowed to settle for 5 min. The microbial cells were separated from the uppermost 50 ml of the settled suspension by a series of low-speed centrifugations, each for 5 min at 650 x *g*. After each centrifugation, the supernatant fluid was decanted and retained, while the pellet was resuspended in fresh 0.1% sodium pyrophosphate. The final pellet was discarded. The five supernatant fluids were then pooled and centrifuged for 20 min at 24,000 x *g* and the cleared supernatant fluid was discarded. The uppermost, usually dark-brown or black, layer of the resulting pellet was removed with a small spatula and suspended in a 0.1% (w/v) ruthenium red (RR) solution in 0.05 *M* sodium cacodylate fubber, pH 7.2, for 2 h to enhance contrast of acidic exopolymers. Fixation was continued for an additional 2-h period in a mixture containing 3.0% glutaraldehyde and 0.1% RR in the cacodylate buffer. After fixation, the samples were rinsed several times in buffer and post-fixed for 2 h in buffered 1% (w/v) OsO_4plus 0.1% RR. Post-fixed samples were rinsed in buffer, enrobed in agar, dehydrated through a graded ethanol series, and embedded in Spurr's low-viscosity epoxy resin (Spurr 1969). The entire fixation, dehydration, and embedding procedure was carried out at room temperature. The epoxy resin was polymerized for 8 h at 70 C.

Thin sections were cut on an LKB Ultratome III ultramicrotome with either glass knives or a Diatome diamond knife. Sections were retrieved on Formvar-coated, 200-mesh, copper EM grids, post-stained in 0.5% (w/v) uranyl acetate dissolved in 50% aqueous methanol followed by 0.4% (w/v) lead citrate. Sections were viewed in a JOEL JEM-100S transmission electron microscope, operating at an accelerating potential of 80 or 100 kV.

Results and Discussion

Enumeration of Subsurface Microorganisms

Numbers of AO-fluorescent microbial cells in all samples from boreholes 6B and 7 were consistently between 1 and 10 million/g dry wt (Table 1). As we observed previously in Oklahoma samples (Ghiorse and Balkwill 1981), the vast majority of microorganisms in the Ft. Polk samples appeared to be bacteria (see discussion below). Bacterial cell counts of samples fixed immediately after sampling were not significant-

ly different from counts of samples that were shipped and stored at 4 C (Table 1), nor were the counts different in samples stored at 4 C for 6 months (not illustrated). These results support our previous conclusion that subsurface samples can be stored at 4 C for considerable periods of time without altering the size of the bacterial population in them (Ghiorse and Balkwill 1981). The numbers of bacteria found in the Ft. Polk samples were strikingly similar to those reported previously in Oklahoma samples, indicating that these subsurface environments contained bacteria at uniformly low population densities.

Slightly elevated numbers (~10 million/g dry wt) were counted in the uppermost sample (1.2 m) and in the deepest sample (5.1 m) from borehole 7 (Table 1). The elevated numbers in the 1.2-m sample correlated with a higher TOC (0.053% at 1.2 m vs. ~0.02% in other samples) and with elevated plate counts (~10^5 CFU/g dry wt), indicating that the higher organic carbon content may have supported a higher bacterial population in this sample. No such correlations were found that would explain higher numbers in the 5.1-m samples.

In general, numbers of CFU were very low on all plating media. In most samples less than 100 CFU/g dry wt, 10,000-fold less than AO-fluorescent cell counts, were detected (Table 1). These results are somewhat different from those reported for the Oklahoma samples in which, in some instances, CFU were only twofold lower than AO-fluorescent cell counts (Balkwill and Ghiorse 1982). Such variations in viable cell counts is not surprising considering the difficulties that might be encountered when cultivating bacteria with unknown nutritional requirements.

Cultivation and Isolation of Subsurface Microorganism

The foregoing result indicated that very few of the bacteria in the Ft. Polk samples could grow on the SEA, PYG, or 1% PYG media employed for plate counts. This implies that either most of the bacteria in these samples were not viable or that the media employed were not adequate to support their growth. The second of these possibilities seems most likely to be correct since small zones of bacterial growth were frequently observed around soil particles on SEA plates. However, the bacteria in these zones did not grow when transferred to fresh media suggesting that unknown factors, possibly including more acidic conditions that apparently exist in the soil, were needed to support their growth.

In one instance, however, a filamentous actinomycete-like bacterium was isolated from the 1.2-m and 5.1-m samples from borehole 7. In addition, filamentous forms were observed in the 1.2-m sample by AO-fluorescence microscopy (Fig. 1c) and filamentous bacteria were detected in the 5.1-m sample by TEM (Fig. 2C). Since actinomycete-like colonies were observed frequently on enumeration plates from these same samples, actinomycetes may be among the few indigenous bacteria in these samples that were capable of growth on SEA and PYG media. These results illustrate the need for further investigations of the growth requirements of bacteria indigenous to subsurface environments. They indicate that novel approaches may be required to resolve the problems of cultivating subsurface bacteria (for discussion of this point see Ghiorse and Balkwill 1981).

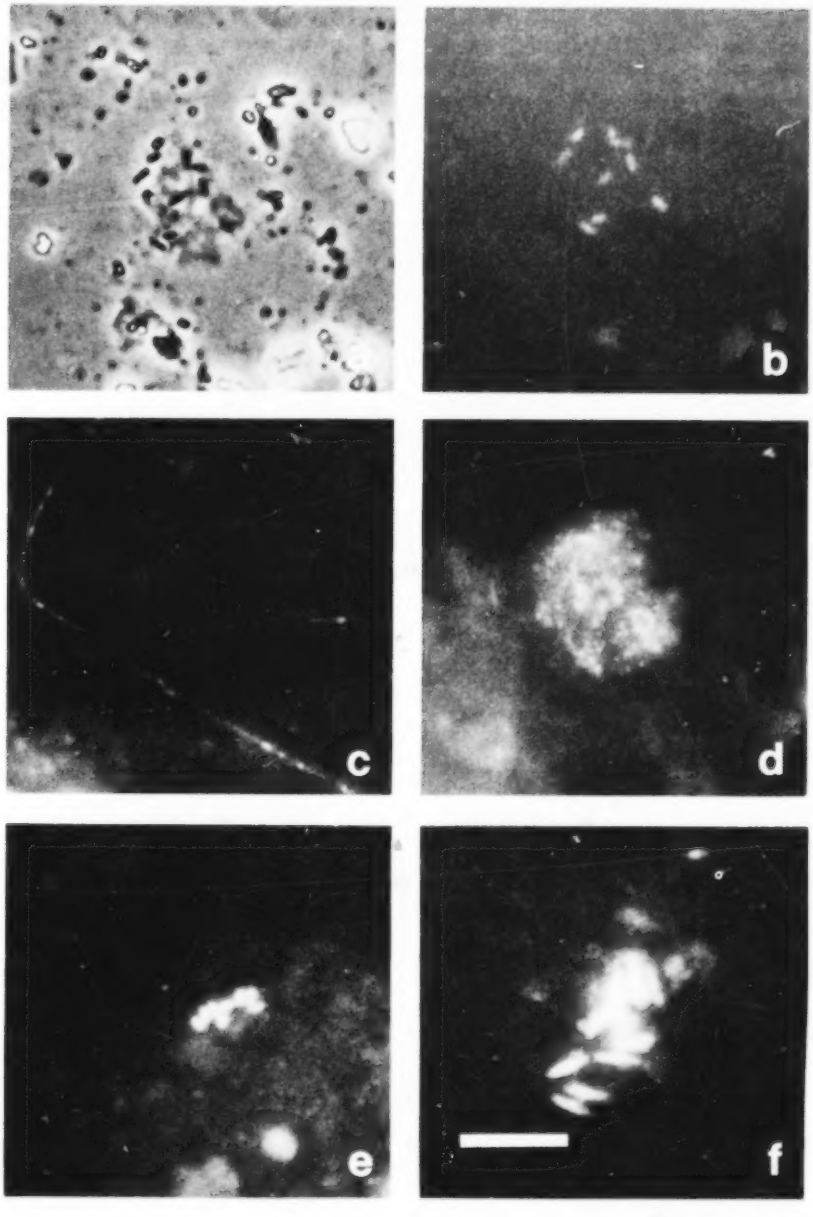

FIG. 1. Light micrographs illustrating various forms of bacterial cells observed in AO-stained subsurface soil smears. (a) A typical field under phase contrast. Borehole 6B, 6.7 m (saturated zone). (b) Same field as shown in (a) but under epifluorescence. Note that only bacterial cells are seen under epifluorescence. (c) A filament and (d) a microcolony. Epifluorescence. Borehole 7, 1.2 m (unsaturated zone). (e) A group of

ovoid cells and (f) rods with tapered ends. Epifluorescence. Borehole 7, 5.1 m (saturated zone). Bacterial cells in micrographs (b-f) fluoresced bright green, usually against a background or orange clay particles. Magnification of all micrographs is 1600X. Bar = 10 μm.

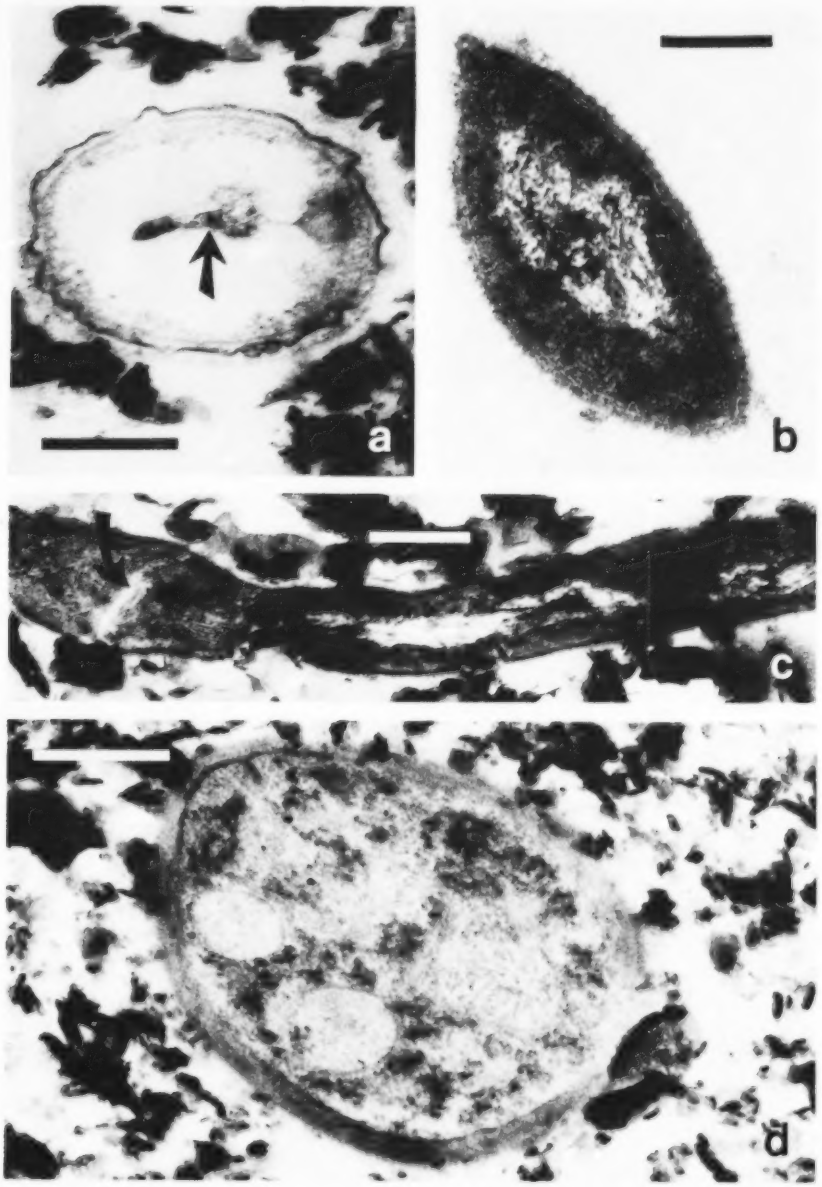

FIG. 2. Electron micrographs of thin-sectioned cells released from samples of Ft. Polk subsurface soil by the blending-centrifugation method. (a) A gram-negative cell with condensed nuclear material (arrow).

Borehole 7, 1.2 m (unsaturated zone). Bar = 0.3 μm. (b) A gram-positive, ovoid cell. Borehole 7, 4.0 m (unsaturated zone). Bar = 0.2 μm. (c) A filamentous, gram-positive cell. Note division septum (arrow). Borehole 7, 5.1 m (unsaturated zone). Bar = 0.3 μm. (d) Possibly a yeast cell. Borehole 6B, 3.7 m (unsaturated zone). Bar = 0.5 μm.

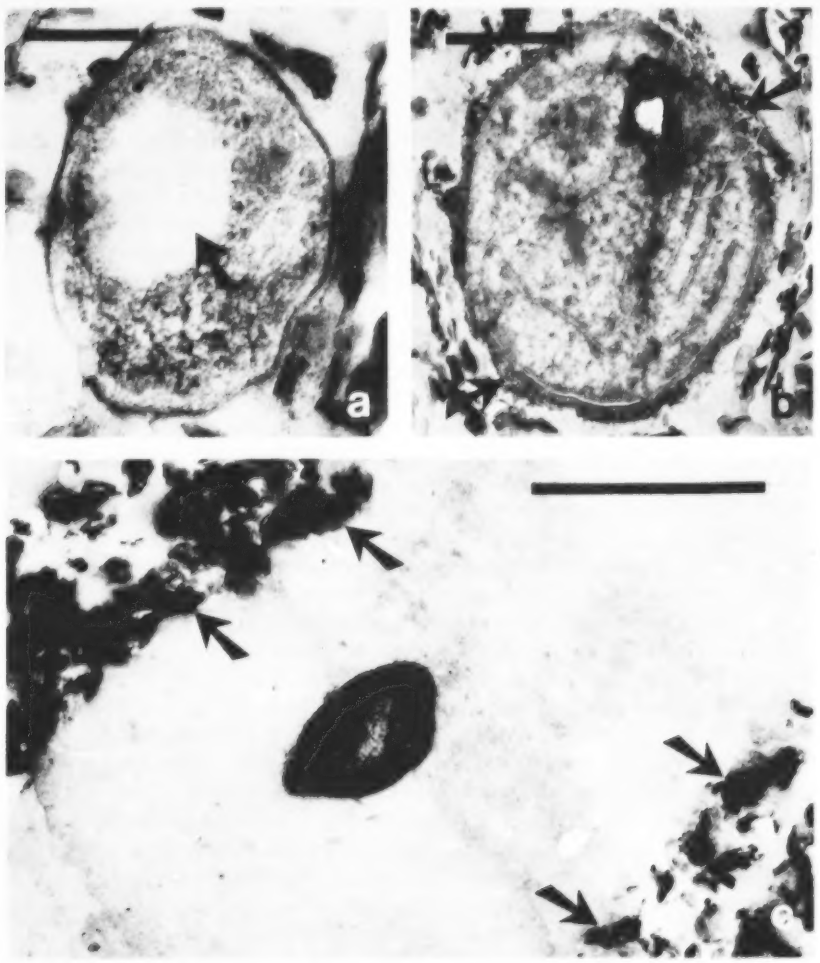

FIG. 3. Electron micrographs of thin-sectioned cells released from samples of Ft. Polk subsurface soil by the blending-centrifugation method. (a) A gram-positive cell with poly-*B*-hydroxybutyrate (PHB)-like inclusion granule (arrow). Borehole 6B, 6.7 m (saturated zone). Bar = 0.2 μm. (b) a gram-negative cell with intracytoplasmic membranes. Note electron-dense, RR-stained glycocalyx material (arrows). Borehole 6B, 5.5 m (unsaturated zone). Bar = 0.5 μm. (c) A bacterial cell surrounded by large amount of glycocalyx material. Note that soil particles (arrows) are excluded by the glycocalyx material. Borehole 7, 4.0 m (unsaturated zone). Bar = 1.0 μm.

Morphological Characterization of Subsurface Microorganisms

Both AO-fluorescence microscopy (Fig. 1) and TEM of thin sections (Figs. 2 and 3) revealed a variety of morphologically distinct microorganisms inhabited the Ft. Polk samples. The combined results of these two techniques leave little doubt that a diverse and potentially active microbial community was present in these samples despite the facts that plate counts were very low and that attempts to cultivate subsurface bacteria met with little success.

Similar to the results with Oklahoma subsurface samples (Balkwill and Ghiorse 1981), small ($> 1\ \mu m$) rods (Fig. 1 b,e) and cocci (Fig. 1d) were seen quite frequently in the Ft. Polk samples. However, these samples also contained a number of distinctive microbial forms that were not observed in the earlier studies. These included ellipsoid cells with pointed ends (Figs. 1f, 2b), narrow filaments (Figs. 1c, 2c), and a few ovoid forms (Fig. 2d) that could have been fungal spores or yeast cells, based on their size and their ultrastructure.

These findings are especially interesting because eukaryotic forms were not detected previously in the Oklahoma samples. Furthermore, biochemical "finger print" analyses of the Ft. Polk samples revealed no detectable polyenoic fatty acids, characteristic of microeukaryotes, in these samples (White et al. 1981). The previous results led to the conclusion that few, if any, eukaryotic microorganisms existed in subsurface environments. It now seems likely that some microeukaryotes may be present in these environments, but in extremely low numbers. Indeed, considering the low level of organic matter found in these samples, one would expect to find forms of life that are physiologically and structurally adapted to low concentrations of nutrients. Dormant forms of eukaryotes might survive in such oligotrophic environments, but oligocarbophilic bacteria would certainly be better adapted to live in them (Poindexter 1981). Thus a paucity of eukaryotic life still appears to be a characteristic feature of oligotrophic subsurface environments.

The consistent observation of groups of cells (microcolonies) with similar structural characteristics in the Ft. Polk samples (Fig. 1d, e, f) and in the Oklahoma samples (Balkwill and Ghiorse 1982; Ghiorse and Balkwill 1981) infers that a portion of the microflora in these samples was actively growing either during, or just prior to, the time of sampling. The presence of cross walls (Fig. 2c) in some subsurface bacteria further supports this inference. This evidence, although circumstantial, supports the idea that many subsurface microorganisms are not dormant or inactive, rather they may grow and divide at reduced rates determined by the amount of nutrients available to them.

The TEM of the Ft. Polk samples also indicated that, similar to the Oklahoma samples, a majority of bacteria released from these samples possessed gram-positive-type cell envelopes (Fig. 2b, c, d; Fig. 3a, b). Bacteria with gram-negative-type envelopes (Fig. 2a) were present; however, gram-negative forms comprised less than one-third of all the bacteria observed by TEM. These results are consistent with the finding of a high proportion of muramic acid compared to lipid A in these samples, indicating an enrichment in gram-positive bacteria (White et al. 1981).

The TEM of thin sections also can provide information regarding the physiological state or nutrient status of cells. For example, poly-*B*-hydroxybutyrate (PHB) storage inclusions should be expected to occur in bacteria adapted to low-nutrient

(oligotrophic) environments (Poindexter 1981). Thus, the presence of PHB-like granules (Fig. 3a) suggests the bacteria containing them were adapted to long-term nutrient limitation, characteristic of oligotrophic environments. These results are enhanced by the previous findings of White et al. (1981) who reported a 20-fold greater concentration of PHB per mole of muramic acid in Ft. Polk subsurface samples than in surface sediment samples, indicating a large proportion of the subsurface bacteria store PHB. Additionally, the observation of bacterial cytoplasm depleted of many normal constituents (Fig. 2a) also suggests that subsurface bacteria exist under nutrient limitation. Such lack of cytoplasmic constituents does not appear to be the result of poor preservation of these bacteria since the fixation procedure used was shown to adequately preserve cultured subsurface bacteria after they were added to samples of subsurface soil (unpubl. observ.).

On the other hand, some of the bacteria observed by TEM appear to be robust in their adaptation to the low nutrient conditions in which they live. This is suggested by the relatively "healthy" ultrastructural appearance of some cells in these samples (Figs. 2b, 3c). In addition, some of the bacteria in these samples contained internal membrane systems (Fig. 3b) reminiscent of the membrane systems in nitrifying or methane-oxidizing bacteria. The presence of internal membrances implies that some bacteria in subsurface environments may be able to use inorganic nitrogen (e.g., NH_4^+, NO_2^-), or CH_4, or even inorganic sulfur compounds as energy sources for growth.

Finally, some bacteria in these samples were surrounded by material that was stained with RR (Fig. 3b, c). Such RR-stained material can be interpreted as a glycocalyx, probably containing acidic polysaccharide, similar to that found surrounding microorganisms in many natural ecosystems (Costerton et al. 1978). Such extracellular polymeric material could aid bacteria in oligotrophic environments by improving their ability to bind to the surfaces of minerals where nutrients may accumulate. Alternatively, the glycocalyx might serve as an extended network for scavenging trace nutrients present at low concentrations in ground water.

Conclusions

The results of this work reveal that unformly low numbers of bacteria populate the shallow, oligotrophic subsurface environments examined. Many of the subsurface bacteria appear to be adapted for survival under nutrient-limited conditions and some appear to be capable of growth under these conditions. In any case, the results suggest that subsurface bacteria exist under low-nutrient stress. Thus, the sparse populations of these bacteria would appear to be at risk.

Since the bacteria in a given subsurface environment may degrade some, but not all, of the organic pollutants commonly found in groundwater (Wilson et al. 1983), many subsurface pollutants would be expected to persist at low levels for extended periods of time. Continued addition of toxic wastes to subsurface environments may cause elevated levels to accumulate in groundwater, eventually inactivating or destroying a large proportion of the subsurface bacteria. In such an eventuality, the probability that a polluted subsurface environment would ever recover from pollution by toxic wastes would be greatly reduced.

ACKNOWLEDGMENTS

This work was supported by Subcontract No. 6931-5 under U.S.E.P.A. Cooperative Agreement No. CR806931-02. The able technical assistance of Josephine Givot, Kathryn Echols, and Leanne Tanouye is gratefully acknowledged. We also thank S. H. Zinder for critically reading the manuscript.

LITERATURE CITED

Alexander, M. 1981. Biodegradation of chemicals of environmental concern. *Science* 211:132-138.

Balkwill, D. L., and W. C. Ghiorse. 1982. Characterization of subsurface microorganisms. Abst. Annual Meeting Am. Soc. Microbiol. N85, p. 192.

Balkwill, D. L., T. E. Ruckinsky, and L. E. Casida, Jr. 1977. Release of microorganisms from soil with respect to electron microscopy viewing and plate counts. *Antonie van Leeuwenhoek J. Microbiol. Serol.* 43:73-81.

Costerton, J. W., G. G. Geesey, and K. J. Cheng. 1978. How bacteria stick. *Sci. Am.* 238:86-95.

Dunlap, W. J., and J. F. McNabb. 1973. Subsurface biological activity in relation to ground water pollution. EPA-6601/273-014, National Environmental Research Center, Office of Research Monitoring U.S.E.P.A., Corvallis, OR.

Dunlap, W. J., J. F. McNabb, M. R. Scalf, and R. L. Cosby. 1977. Sampling for organic chemicals and microorganisms in the subsurface. EPA-600/2-77-176, National Environmental Research Center, Office of Research Monitoring U.S.E.P.A., Corvallis, OR.

Ghiorse, W. C., and D. L. Balkwill. 1981. Microbiological characterization of subsurface environments. *Proc. First Int. Conf. on Ground Water Quality Research*. Rice University, Houston, TX. October 1981.

McNabb, J. R., and W. J. Dunlap. 1975. Subsurface biological activity in relation to ground-water pollution. *Ground Water* 13:33-44.

Olson, G. J., W. C. Dockins, G. A. McFeters, and W. P. Iverson. 1981. Sulfate-reducing and methanogenic bacteria from deep aquifers in Montana. *Geomicrobiol. J.* 2:327-340.

Poindexter, J. A. 1981. Oligotrophy: Fast and famine existence. *Adv. Microb. Ecol.* 5:63-89.

Spurr, A. R. 1969. A low viscosity epoxy resin embedding medium for electron microscopy. *J. Ultrastruct. Res.* 26:31-43.

Trolldenier, G. 1973. The use of fluorescence microscopy for counting soil microorganisms. *In* T. Rosswall, ed. Modern methods in the study of microbial ecology. *Ecol. Bull.* (Stockholm) 17:103-108.

White, D. C., J. S. Nickels, H. J. Parker, R. H. Findlay, M. J. Gehron, G. A. Smith, and R. F. Martz. 1981. *Proc. First Int. Conf. on Ground Water Quality Research.* Rice Univesity, Houston, TX, October 1981.

White, D. C., H. F. Fredrickson, M. J. Gehron, G. A. Smith, J. H. Parker, J. S. Nickels, and R. H. Findlay. 1983. The ground water aquifer microbiota: Biomass, community structure, and nutritional status. *Dev. Ind. Microbiol.* 24:189-199.

Willis, C. J., G. H. Elkan, E. Horvath, and K. R. Dail. 1975. Bacterial flora of saline aquifers. *Ground Water* 13:406-409.

Wilson, J. T., J. F. McNabb, B. H. Wilson, and M. J. Noonan. 1983a. Biotransformation of selected organic pollutants in ground water. *Dev. Ind. Microbiol.* 24:225-233.

Wilson, J. T., J. F. McNabb, D. L. Balkwill, and W. C. Ghiorse. 1983b. Enumeration and characterization of bacteria indigenous to a shallow water-table aquifer. *Ground Water* 21: in press.